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Repair of Global Regulators in *Staphylococcus aureus* 8325 and Comparative Analysis with Other Clinical Isolates▽†

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The pathogenicity of *Staphylococcus aureus* strains varies tremendously (as seen with animals). It is largely dependent on global regulators, which control the production of toxins, virulence, and fitness factors. Despite the vast knowledge of staphylococcal molecular genetics, there is still widespread dispute over what factors must come together to make a strain highly virulent. *S. aureus* NCTC8325 (RN1 and derivatives) is a widely used model strain for which an incomparable wealth of knowledge has accumulated in the almost 50 years since its isolation. Although RN1 has functional *agr*, *sarA*, and *sae* global regulators, it is defective in two regulatory genes, *rsbU* (a positive activator of SigB) and *tcaR* (an activator of protein A transcription), and is therefore considered by many to be a poor model for studies of regulation and virulence. Here, we repaired these genes and compared the resulting RN1 derivatives with other widely used strains, Newman, USA300, UAMS-1, and COL, plus the parental RN1, with respect to growth, extracellular protein pattern, hemolytic activity, protein A production, pigmentation, biofilm formation, and mouse lethality. The *tcaR*-repaired strain, showed little alteration in these properties. However, the *rsbU*-repaired strain was profoundly altered. Hemolytic activity was largely decreased, the exoprotein pattern became much more similar to that of typical wild-type (wt) *S. aureus*, and there was a surprising increase in mouse lethality. We note that each of the strains tested has a mutational alteration in one or more other regulatory functions, and we conclude that the repaired RN1 is a good model strain for studies of staphylococcal regulation and pathobiology; although strain Newman has been used extensively for such studies in recent years, it has a missense mutation in *saeS*, the histidine kinase component of the *sae* signaling module, which profoundly alters its regulatory phenotype. If this mutation were repaired, Newman would be considerably improved as a model strain.

As a classical, dangerous, and universal human pathogen, *Staphylococcus aureus* has aroused continuing interest in its epidemiology, pathogenesis, and antibiotic resistance and other features of its pathobiology. This interest has led, concomitantly with the development of bacterial molecular biology, to the development of a model strain for the analysis of staphylococcal molecular genetics in relation to the pathogenicity of the organism. This strain, NCTC8325, was isolated in 1960 from a sepsis patient and utilized as the propagating strain for phage 47 of the international phage typing system. It was originally chosen for research owing to its sensitivity to all known antibiotics and was initially used primarily for studies of antibiotic resistance transfer and carriage by plasmids (32). It is designated RN1 in the Novick lab strain collection and is presently maintained by the Central Public Health Laboratory, Colindale, London, United Kingdom, by the ATCC and by the recently established Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) collection. RN1 is the progenitor of a large series of mutant and plasmid-containing derivatives that have been used for the development of molecular tools and for the study of genetic regulation (31). The most important of these strains are maintained by NARSA; their pedigree is shown in Fig. 1.

The 2.8-Mb 8325 genome has been sequenced (GenBank accession no. CP000253) and is syntenic with the genomes of the other sequenced strains (18 at last count). RN1 carries three prophages (Φ11, Φ12, and Φ13) (21, 30). It is *agr* group 1 and *agr*$^+$, and it produces alpha-, delta-, and gamma-hemolysins, but no beta-hemolysin, as prophage Φ13 is integrated in *hlb* (10). The 8325 lineage remains the standard for basic research on gene regulation, plasmid replication, prophage behavior, cell wall structures, membrane composition, physiology, and antibiotic resistance. Much of the research on fitness traits and virulence factors, such as toxins, adherence to host cells, invasion, host response, or interaction with the immune system, has also been performed with 8325 derivatives. RN1 is, therefore, the staphylococcal counterpart of *Escherichia coli* K-12. We note that for genetic studies with bacteria other than the standard model organisms, it is convenient to perform genetic manipulations, cloning, etc., in *E. coli* K-12 and then return the products to the organism under study. However, *S. aureus* does not accept DNA from *E. coli*, owing to restriction incompatibilities and possibly other barriers. Two *S. aureus* mutants that accept *E. coli* DNA and are therefore in very wide use in staphylococcal research are SA113 and RN4220, both isolated following nitrosoguanidine mutagenesis (MNNG) and

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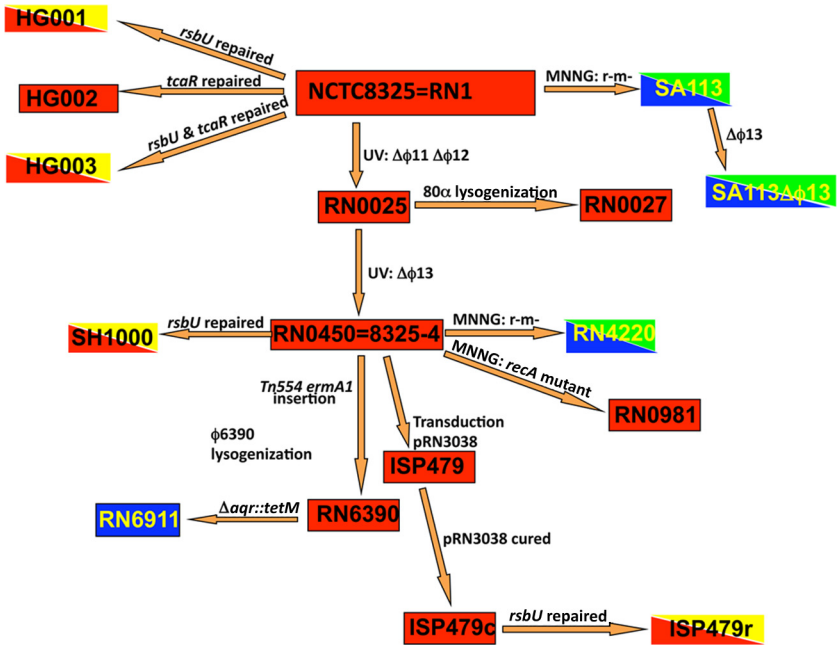

FIG. 1. NCTC8325 pedigree. RN1 (11-bp deletion in *rsbU*, point mutation in *tcaR*, three prophages, Φ11, -12, and -13) and derivatives HG001 (*rsbU* restored), HG002 (*tcaR* restored), HG003 (*rsbU* and *tcaR* restored), NCTC8325-4 (11-bp deletion in *rsbU*, cured of three prophages), SH1000 (*rsbU* restored, cured of three prophages), ISP479c (11-bp deletion in *rsbU*, cured of three prophages, spontaneous cadmium-resistant revertant), ISP479r (*rsbU* restored, cured of three prophages, spontaneous cadmium-resistant revertant), SA113 (*rsbU tcaR agr* mutant and three prophages, Φ11, -12, and -13), SA113ΔΦ13 (*rsbU tcaR agr* mutant and two prophages, Φ11 and -12). Red, agr^+; blue, *agr* mutant; yellow, $sigB^+$ ($rsbU^+$); green, r^- m^- (restriction-modification mutant) (see text for details).

both *agr* defective (22, 24). Other important strains are 8325-4 (also known as RN0450) (UV cured of all 3 prophages [30] and therefore useful for phage propagation) and ISP479c, a derivative of 8325-4 containing a thermosensitive replication-defective mutant of pI258, which is an effective donor of transposon Tn*551*, carrying erythromycin resistance (5).

For the analysis of staphylococcal regulation and pathogenesis, however, the utility of RN1 and its derivatives has been questioned, on the one hand, because of their defect in *rsbU* (6) (see below), despite the fact that RN1 is a human sepsis isolate. On the other hand, as staphylococcal pathogenesis is multifactorial and as strains vary widely in their possession and/or expression of virulence factor genes and their regulation, no one strain can currently be regarded as a workhorse strain for studies of staphylococcal pathobiology, and a few well-characterized clinical strains with a diversity of virulence genes and virulence gene expression, namely, Newman, COL, UAMS-1, N315, and USA300, are currently in use for this purpose.

Because of the extensive experience with the 8325 lineage, its high level of characterization, and its utility for studies of molecular genetics, it has seemed worthwhile to bring its regulatory structure into line with other model strains, for studies of regulation and of pathogenesis. Thus, two different laboratories have "repaired" the *rsbU* deletion, in 8325-4 (20), generating SH1000, and in ISP497c (38), generating ISP479r. Since most native *S. aureus* strains are multiply lysogenic, it is our view that a better starting strain would be RN1, rather than 8325-4 or its derivatives, which have not only been cured of prophages but have been subjected to successive cycles of UV irradiation, which could have introduced cryptic mutations with unknown effects. Accordingly, we have introduced into RN1 a new markerless repair of *rsbU* and have also repaired the mutation in *tcaR*. In this report, we compare native RN1 with these repaired strains and with the other commonly used strains listed above, for expression of several key genes, for overall exoprotein patterns, for hemolysin and staphyloxanthin production, for biofilm formation, and for mouse lethality.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains used and constructed are listed in Table 1. Bacteria were grown in basic medium (BM) (1% tryptone [Gibco BRL Life Technologies GmbH, Eggenstein, Germany], 0.5% yeast extract [Gibco BRL, Eggenstein, Germany], 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose).

Construction of HG001 (NCTC8325 *rsbU*$^+$), HG002 (NCTC8325 *tcaR*$^+$), and HG003 (NCTC8325 *rsbU*$^+$ *tcaR*$^+$). The replacement of the 11-bp deletion in the *rsbU* gene in RN1 and the repair of the A → T transversion in the *tcaR* gene were carried out by homologous recombination using pMAD*rsbU* and pMAD*tcaR* vectors with blue-white screening on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates as described in reference 2. The pMAD*rsbU* vector contained a 3-kb fragment of the complete *rsbU-rsbV-rsbW* region and part of *sigB*, amplified with primers OL-78 (TATCTTACCAATCTTTGATAATCTCG ATAAC) and OL-79 (GCTCTAGAGTTCAAGACATTAGATG) by PCR from the *rsbU*$^+$ Newman DNA (20, 38). The successful replacement of the 11-bp deletion in *rsbU* was verified by both DNA sequencing (Licor) with the primer *rsbU*up (AGTGTGAGAAATACACTGACG) and PCR with the primers *rsbU*up and *rsbU*down (CTACCAATCTTTGATAATCTCG). The pMAD*tcaR* vector contained a 3.4-kb fragment of intact *tcaR*, and the *tcaR* gene up- and downstream flanking regions were amplified from Newman DNA with primers tcaR1 (TATAGAAGGATCCAGTAATGTGCCAGATGGGC) and tcaR2 (CAAGC TCCCATGGTTTATCTACTTGCTTACCG). Successful repair of *tcaR* was verified by DNA sequencing using the primer *tcaR*down (TGATGACTTCTAATA CTTGCC). The double repair in HG003 (NCTC8325 *rsbU*$^+$ *tcaR*$^+$) was carried out in the same way.

TABLE 1. Investigated *S. aureus* strains

Strain	Genotype/description	Phenotype[a]	First isolation/ publication	References
Test strains				
8325 (NCTC8325 = RN1)	*rsbU tcaR*	MSSA, Agr^+	1960	30
HG001 (RN1 derivative)	*rsbU* repaired, *tcaR*	MSSA, Agr^+	This study	This study
HG002 (RN1 derivative)	*rsbU*; *tcaR* repaired	MSSA, Agr^+	This study	This study
HG003 (HG001 derivative)	*rsbU* and *tcaR* repaired	MSSA, Agr^+	This study	This study
SA113ΔΦ13 (RN1 derivative)	*rsbU agr tcaR*	MSSA, Agr^-	1976	22; this study
Newman	*saeRS* constitutively expressed by SaeS T53C substitution, *fnbA* and *fnbB* mutated	MSSA, Agr^+	1952	12
USA300		CA-MRSA, Agr^+	2000	29
UAMS-1	*sarT sarU hla*	MSSA, Agr?	1995	16
COL	*agr*, lacks ΦSA3, which integrates in *hlb*	MRSA, Agr^-	1961	13
Control strains				
Newman Δ*hla*::*erm*	α-Hemolysin gene (*hla*) deleted			17
SA113 Δ*spa*::*erm*	Protein A gene (*spa*) deleted			This study
RN6390 Δ*agr*::*tet* (RN6911)	*agr* operon deleted			23
RN1 *agr* mutant	Spontaneous *agr* mutant with Hla-negative phenotype and overproduction of Spa			This study

[a] MSSA, methicillin-sensitive *Staphylococcus aureus*; Agr?, phenotype not clear.

Staphyloxanthin production. Overnight cultures were diluted in BM and plated on BM agar and on sheep blood agar (SBA). Staphyloxanthin production is indicated by yellow-orange pigmentation of colonies on BM agar incubated at 37°C for 48 h.

Hemolysis activity of the *S. aureus* strains. Hemolytic activity is indicated on SBA and was determined in two ways. On the one hand, hemolytic activity was determined by cross-streaking perpendicularly to RN4220, which produces only beta-hemolysin (39); in the absence of cold shock, this test can usually identify the two staphylococcal hemolysins: alpha- and delta-hemolysins (40) (beta-hemolysin enhances lysis by delta-hemolysin but inhibits lysis by alpha-hemolysin [14] [illustrated in Fig. 2B]). On the other hand, hemolytic activity was determined on SBA, after incubation at 37°C for 24 h followed by cold shock at 4°C for 12 h (beta toxin is also known as the hot-cold toxin because of its unique activity on sheep blood agar plates; at 37°C, beta toxin interacts with sheep red blood cells but does not lyse them; if the red cells are then placed at 4°C, the cells lyse; this is observed as a lack of hemolysis on blood agar plates at 37°C and then complete hemolysis at 4°C [illustrated in Fig. 2C]).

Exoprotein pattern. The *S. aureus* strains were aerobically grown (rotary shaker) in BM at 37°C for 2.5 h and 7 h. All cultures were diluted with BM to the same optical density at 578 nm (OD_{578}) of 1.0 after 2.5 h of incubation and of 6.0 after 7 h of incubation and centrifuged. The exoproteins in the supernatant were concentrated with StrataClean resin (Stratagene) run in SDS-PAGE (12%) and stained with Coomassie blue (0.1% Coomassie brilliant blue R250, 10% acetic acid, 50% methanol).

Identification of proteins by mass spectrometry (MS). Protein bands were excised from gels. In-gel digestion with trypsin and extraction of the peptides were done using the Ettan spot handling workstation (Amersham Biosciences). Peptide masses were determined with a mass spectrometer (MALDI-TOF Proteome Analyzer 4700; Applied Biosystems, Foster City, CA). Peptide mass fingerprints were analyzed using GPS Explorer with integrated MASCOT software (Matrix Science). Criteria for identification of a protein were a MASCOT score above 49, a replicated result of the identification at the same position, and an observed molecular mass that was close to that of the predicted protein apart from protein fragments. For multiple identifications in one protein band, all secure identifications according to these criteria were indicated.

Western blotting. Western blot analysis was carried out as described in the protocol in the *QIAexpress Detection and Assay Handbook* (Qiagen), with a semidry transfer procedure. Supernatants of all *S. aureus* strains were isolated after 2.5 or 7 h, adjusted to an OD_{578} of 1.0 or 6.0, respectively, with BM, centrifuged, and concentrated with StrataClean resin (Stratagene). Alpha-hemolysin was detected with rabbit anti-staphylococcal alpha-hemolysin antiserum (1:20,000) (Sigma-Aldrich) and goat alkaline phosphatase (AP)-conjugated anti-

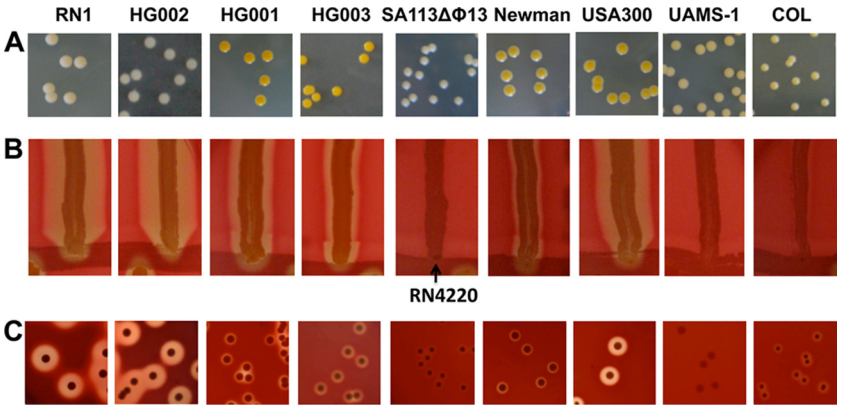

FIG. 2. Staphyloxanthin production and hemolysin activities of the *S. aureus* strains. (A) Pigmentation (staphyloxanthin production) and colony size of *S. aureus* RN1 and derivatives HG002, HG001, HG003, and SA113ΔΦ13 and the *S. aureus* clinical isolates Newman, USA300, UAMS-1, and COL on BM plates. (B) Hemolytic activities (no cold shock applied) determined by cross-streaking perpendicularly to RN4220, which produces only beta-hemolysin, which enhances lysis by delta-hemolysin but inhibits lysis by alpha-hemolysin. (C) Hemolysin activity on sheep blood agar (SBA) after cold shock at 4°C for 12 h.

rabbit IgG (1:20,000) (Sigma-Aldrich). Protein A was visualized with goat anti-staphylococcal Spa antiserum (1:20,000) (Rockland) and rabbit AP-conjugated anti-goat IgG (1:20,000) (Sigma-Aldrich). Chemiluminescence detection was performed with BCIP (5-bromo-4-chloro-3-indolylphosphate)/NBT (Nitro Blue Tetrazolium) solution.

RNA isolation and Northern blot hybridization. *S. aureus* cells were isolated after 2.5 and 5 h and adjusted to an OD_{578} of 1.0 or 4.0 with BM. A total of 10 ml of cells at an OD_{578} of 1.0 and 2 ml cells at an OD_{578} of 4.0 were centrifuged and lysed in 1 ml Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5-ml zirconia/silica beads (0.1-mm diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of Trizol. Digoxigenin-labeled RNA probes of the corresponding genes were prepared by *in vitro* transcription with T7 RNA polymerase by using a PCR fragment as a template (15). The PCR fragment was generated by using chromosomal DNA of SA113. The reverse oligonucleotides contain the T7 RNA polymerase recognition site sequence at the 5′ end. For Northern blot analyses, equal amounts of total RNA (10 μg) were separated under denaturing conditions in a 1% agarose gel containing 20 mM MOPS (morpholinepropanesulfonic acid), 5 mM sodium acetate, 1 mM EDTA, 1.85% formaldehyde, pH 7.0. The gel was blotted onto a nylon membrane with 20× SSPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA [pH 7.4]) by using a vacuum blotter. After 4 h, RNA was fixed at the membrane by UV cross-linking for 1 min. The membrane was stained with methylene blue to visualize 16 and 23S rRNA bands in order to control the successful blotting and the existence of the same amount of total RNA in each lane.

The digoxigenin-labeled RNA probes were used for gene-specific hybridization according to the manufacturer's instructions (Roche, Mannheim, Germany). The detection of the hybridization signals was performed using the Lumi-Film chemiluminescence detection film (Roche, Mannheim, Germany).

Biofilm assay. An overnight culture was diluted 1:200 in fresh Trypticase soy broth (TSB) (0.5% glucose). A total of 200 μl was filled in 96-well microtiter plates and incubated for 24 h at 37°C without shaking. The supernatant was discarded, the plate was dried, and the cells were stained with 0.1% safranin (18).

Mouse sepsis model. All animal studies were approved by the Animal Care and Experimentation Committee of the district government of Unterfranken, Germany, and conformed to University of Würzburg guidelines. Female BALB/c mice (16 to 18 g) were purchased from Charles River, Sulzfeld, Germany, housed in polypropylene cages, and received food and water *ad libitum*. *S. aureus* isolates were cultured for 18 h in BM, washed three times with sterile 0.9% NaCl, and suspended in sterile 0.9% NaCl to 5.0E+08 and 1.0E+08 CFU/100 μl. To control viable cell counts, appropriate dilutions were plated on BM agar. Mice were inoculated with 100 μl of the *S. aureus* strains or with sterile NaCl via the tail vein. The experiments were performed with 6 mice per strain and a control. Criteria for determining morbidity/sickness included body weight, ruffled fur, hunched posture, decreased activity, and labored breathing. Animals were sacrificed if they met the following criteria: (i) loss of at least 20% of body weight, (ii) loss of at least 15% of body weight and ruffled fur; (iii) loss of at least 10% of body weight and hunched posture, and (iv) mortality (mice that meet these criteria are near mortality and die if not sacrificed; therefore, near mortality was scored as death). All mice were sacrificed by 120 h. The statistical significance of mortality studies was determined using the Wilcoxon rank test.

RESULTS

Construction of HG001 (NCTC8325 *rsbU*$^+$), HG002 (NCTC8325 *tcaR*$^+$), and HG003 (NCTC8325 *rsbU*$^+$ *tcaR*$^+$). As noted, strains of the 8325 lineage carry a deletion in *rsbU*. We repaired this deletion in RN1 by homologous recombination using a pMAD vector (2) in which a 3-kb fragment of the *rsbU-rsbV-rsbW-sigB* region was cloned from strain Newman. The resulting strain was named HG001 (NCTC8325 *rsbU*$^+$). As also noted, RN1 also carries a point mutation in *tcaR* that leads to a truncated TcaR regulator. The mutated *tcaR* gene was also repaired by homologous recombination using a pMAD vector carrying a 3.4-kb fragment containing the *tcaR* gene from strain Newman and its up- and downstream flanking regions. The repaired strain was designated HG002 (NCTC8325 *rsbU tcaR*$^+$). A derivative with both mutations repaired, HG003 (NCTC8325 *rsbU*$^+$ *tcaR*$^+$) was constructed in the same way, using the pMAD*tcaR* vector with HG001. These were markerless replacements, confirmed by DNA sequencing, in which the consensus sequences, corresponding to those of strain Newman, were restored. These constructs were then compared to each other and to various other frequently used clinical *S. aureus* isolates.

Comparison of HG003 with other *S. aureus* strains. HG003 (*rsbU*$^+$ *tcaR*$^+$) was compared with its parent strain, RN1 (*rsbU tcaR*), the single-gene-repaired derivatives HG001 (*rsbU*$^+$ *tcaR*) and HG002 (*tcaR*$^+$ *rsbU*), and SA113ΔΦ13 (*rsbU agr tcaR*), as well as with the above-mentioned clinical *S. aureus* isolates, Newman, COL, USA300, and UAMS-1.

Staphyloxanthin formation. In contrast to RN1 and HG002, the colonies of the *rsbU*-repaired strains HG001 and HG003 showed a pronounced yellow-orange pigmentation on BM agar plates (Fig. 2A), corroborating the earlier findings that the carotenoid operon *crtOPQMN* is under positive transcriptional control by SigB (6, 34). Newman and USA300 also form orange colonies, consistent with their possession of an intact *sigB* operon. COL and UAMS-1 are reportedly SigB positive (8, 25) but form only slightly yellow colonies, indicating that staphyloxanthin is weakly produced.

Hemolysis activity. Erythrocytes from sheep blood can be hydrolyzed by various hemolysins, including alpha-, beta-, gamma-, and delta-hemolysins; however, it is difficult to attribute a specific hemolysin to the hemolytic activity. Only gamma-hemolysin is not detectable on blood agar plates, as its activity is inhibited by agar (44). Hemolysis was tested on SBA in two ways. By streaking strains perpendicular to RN4220, which produces only beta-hemolysin, one can usually identify (in the absence of cold shock) alpha- and delta-hemolysins. The strains RN1, HG002, HG001, HG003, Newman, and USA300 produce alpha- and delta-hemolysins, indicated by increased hemolytic activity when the test strains merge with RN4220 (beta-hemolysin of RN4220 boosts activity of delta-hemolysin and suppresses alpha-hemolysin) (Fig. 2B). SA113ΔΦ13 and COL showed no alpha- or delta-hemolysis because SA113 is definitely *agr* negative and COL is most likely affected in *agr*, as indicated by the very low RNAIII transcription (see Fig. 4B). Application of cold shock (Fig. 2C) shows that only SA113ΔΦ13 and COL showed weak beta-hemolysin activity because they both have lost a prophage (Φ13 and ΦSA3, respectively) that is normally integrated in the *hlb* gene (11). Again, the hemolysis of RN1, HG002, HG001, HG003, Newman, and USA300 is due to alpha- and delta-hemolysins. The high hemolytic activity of RN1 and HG002 can be explained by the defect in the *rsbU* gene, as it has been shown that mutation of *sigB* elevates expression of both *agr* and *sarA* (6), which in turn upregulates the alpha-hemolysin gene (*hla*) (9). Consequently, in the *rsbU*-repaired strains, HG001 and HG003, the hemolytic zone is small. The high hemolytic activity of USA300 is very likely not due to a defect in the *sigB* operon, as pigmentation is well expressed; there must be other reasons. Newman showed, like HG001 and HG003, weak hemolysis, suggesting that *sigB* is intact. UAMS-1 showed no hemolysis. One reason is that this strain harbors a mutation in the *hla* gene (8); the other reason is that RNAIII transcription comes very late (not shown), and it has been reported that late RNAIII transcription is associated with failure to translate delta- and alpha-hemolysin (39).

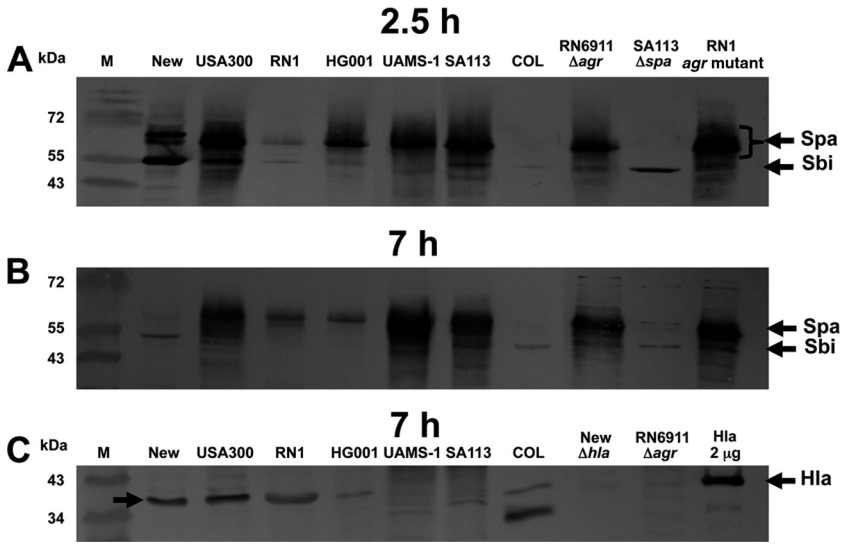

FIG. 3. Western blot for Spa (protein A), Sbi (IgG-binding protein), and Hla (alpha-hemolysin). Western blot with anti-Spa antiserum with supernatant after 2.5-h cultivation (A) and supernatant after 7-h cultivation (B). SA113 *spa* mutant served as negative control. (C) Western blot with anti-Hla antiserum with culture supernatant of a 7-h culture. Purified Hla (2 μg) served as control.

Alpha-hemolysin (Hla) production. As Hla plays an essential role in hemolysis and virulence in *S. aureus*, we compared Hla production levels in all strains by Western blotting with specific Hla antiserum (Fig. 3C). Newman, USA300, and RN1 showed the highest Hla content, while HG001 and COL produced less Hla; currently, we have no explanation for the lower cross-reacting band seen in COL. No Hla was detectable in the *agr* mutants RN6911 and SA113ΔΦ13, as well as in the *hla*-mutated strains UAMS-1 (8) and Newman Δ*hla*. Most of the background bands in the Western blots of SA113ΔΦ13 and UAMS-1 were most likely due to Spa. On the whole, the Western blot results correlated quite well with the hemolytic activity on sheep blood agar (Fig. 2B and C); only the strong Hla band of Newman does not correspond to the small hemolysis zone.

Growth rate. Growth rate was determined as doubling time (min) during exponential growth phase in BM. USA300 (a community-acquired methicillin-resistant *S. aureus* [CA-MRSA]) and the osteomyelitis isolate UAMS-1 showed the shortest doubling times, 32 and 35 min, respectively (Table 2). RN1 and the repaired HG001 to -3 strains, as well as the Newman strain, had doubling times in the range of 40 to 46 min. At the upper end of the range were SA113ΔΦ13 and COL, with doubling times of 54 and 64 min. The lower growth rate of COL and SA113ΔΦ13 is also indicated by the much smaller colonies on both BM agar and, in an even more pronounced manner, on sheep blood agar (Fig. 2). The colony diameters of SA113ΔΦ13 and COL on BM agar are only 72% and 63%, respectively, those of Newman, USA300, UAMS-1, and RN1 derivatives, which all exhibit comparably large colony sizes. On sheep blood agar the difference in the colony sizes of COL and SA113ΔΦ13 is even more pronounced: only 50% compared to the other strains. The genetic basis for these differences in growth rate is unknown. In any case, there was no strong correlation between growth rate and mouse lethality (see below).

TABLE 2. Growth rates of *S. aureus* strains

Strain	Avg[a]
USA300	32 ± 2
UAMS-1	35 ± 2
RN1 (8325)	40 ± 2
HG002	40 ± 3
HG001	43 ± 2
HG003	43 ± 3
Newman	46 ± 2
RN1 *agr* mutant	49 ± 2
SA113ΔΦ13	54 ± 3
COL	64 ± 3

[a] Doubling time (min). Values are the means ± standard deviations of four independent growth curves.

Protein A and Sbi in culture supernatants. Protein A was determined by Western blotting with anti-protein A antiserum. The samples were taken from concentrated culture supernatants and from cells treated with lysostaphin, which releases the cell wall-anchored form. As the relative amounts of Spa in lysostaphin-treated samples or in culture supernatants were comparable, we show here only the results with the culture supernatants. Supernatants were harvested after 2.5 h (Fig. 3A) and 7 h (Fig. 3B) of growth. The highest Spa content was found in the culture supernatants of the *agr* mutants SA113ΔΦ13 and 8325-4 Δ*agr* (RN6911) and in a spontaneous *agr*-defective mutant of RN1. It is well-known that these frequently arise in frozen stock cultures. UAMS-1 and USA300 also produced Spa at high levels, which is unusual for *agr*$^+$ strains. Indeed, RNAIII transcript analysis revealed that with USA300 the transcript level was highest, suggesting an *agr*$^+$ genotype (Fig. 4B). In UAMS-1, the RNAIII transcript is decreased and comes late, which is reportedly associated with no *hld* and comparatively high *spa* transcription (40). This is in good correlation with the absence of delta-hemolysin activity (Fig. 2B) and high *spa* transcript level (Fig. 4A). RN1 produces comparatively little Spa in both 2.5- and 7-h culture. In HG001,

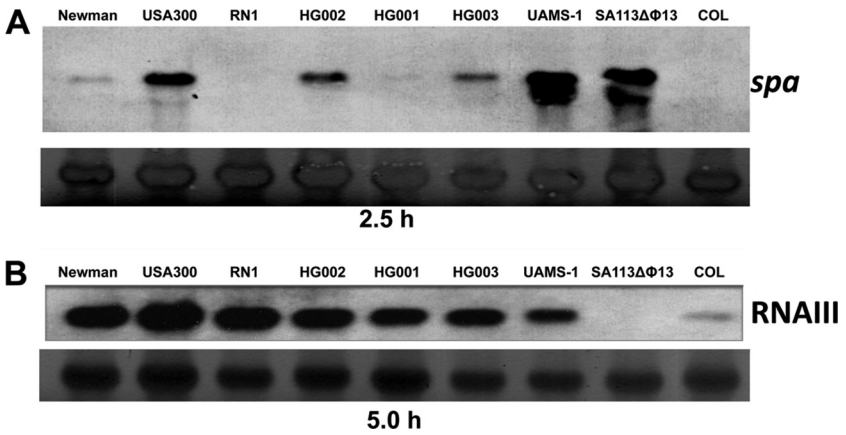

FIG. 4. Northern blot analysis. (A) Northern blot of *spa*. Total RNA (10 μg) isolated from 2.5-h cultures was hybridized with digoxigenin-labeled *spa* PCR fragments. (B) Northern blot of RNAIII. Total RNA (5 μg) isolated from 5-h cultures was hybridized with digoxigenin-labeled RNAIII PCR fragments. The 16S rRNA detected by methylene staining is shown below as a quantitative control.

more Spa was present in the early growth phase at 2.5 h than in the 7-h culture. The Spa content in the *tcaR*-repaired strains HG002 and HG003 (not shown) was slightly increased compared to that of HG001. Newman was peculiar, insofar as Spa was detectable as a double band after 2.5 h, but after 7 h almost no Spa band was visible in the Western blot. We doubt that this signal is due to Spa, as in the lysostaphin-released sample there was very little Spa detectable (not shown). Spa was not detectable in either COL or in the SA113 Δ*spa* mutant (negative control).

Sbi is another IgG-binding protein that exhibits an IgG-binding specificity similar to that of Spa (45); although it lacks the LPXTG motif, it is surface located (46). Its IgG-binding domain is homologous to that of Spa, and therefore, α-Spa antibodies can recognize Sbi. Sbi was detectable in all strains, with a general tendency to be present at a higher level early in growth. This was particularly pronounced in Newman (Fig. 3A and B).

Level of *spa* transcript. SA113ΔΦ13 (*agr* negative), UAMS-1, and USA300 revealed the highest levels of *spa* transcript in the Northern blot (Fig. 4A). This result correlated quite well with the amount of Spa in Western blots. In the *tcaR*-repaired strains HG002 and HG003, *spa* transcript was increased compared to HG001. This result is consistent with published data showing that TcaR (a putative MarR-like regulator) upregulates *sarS*, which is a positive activator of *spa* transcription (28). USA300 and UAMS-1 show high levels of Spa and *spa* transcript similar to those of the *agr* mutant SA113ΔΦ13. Indeed, Northern blot analysis for RNAIII suggests that *agr* is functional in USA300 and UAMS-1 (Fig. 4B). Newman revealed low *spa* transcript levels, and RN1 and COL no *spa* transcript levels.

Extracellular protein patterns. The extracellular protein pattern of a 7-h culture (stationary growth phase) is shown in Fig. 5. The most prominent bands were cut out from the gel, and the proteins contained were analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Table 3). Some bands were composed of more than one protein.

The most prominent protein bands in strain Newman were N1 (major autolysin Atl), N2 (Atl and lipase Lip), N3 (coagulase Coa, Lip, and MapW), N4 (Atl, MapW, Coa, and a hypothetical protein [HP] similar to sulfatase family protein), N5 (IgG-binding protein Sbi), N6 (gamma-hemolysin chain II precursor HlgA), N7 (thermonuclease Nuc), and N8 to N11 (proteins similar to fibrinogen-binding protein Fib). Some of the Fib bands represent degradation products. Only N8 (extracellular fibrinogen-binding protein Efb) and N9 (gene product of NWMN_1066, Fib) appear to represent the intact gene product. In USA300, the most prominent bands were U1 (composed of lipase and Spa, U2 [major autolysin Atl]), U3 (first band consists of lipase and elongation factor Tu [EF-TU], and second band of lipase and major autolysin [Atl]), U4 (glycerol ester hydrolase, Geh), U5 (LukF-PV), U6 (LukS-PV and Lip), U7 (hypothetical protein), U8 (thermonuclease Nuc), U9 (cysteine proteinase staphopain, Stp, which is preferentially expressed in USA300), and U10 (a hypothetical protein, SAUSA300_pUSAO10004, and degradation products of Nuc and Stp).

The most prominent protein bands in RN1 were T1 (Lip), T2 (Atl and HP similar to sulfatase family protein), T3 (upper and lower bands represent lipase protein Lip), T4 (glycerol ester hydrolase Geh), T5 (gamma-hemolysin component B HlgB, HlgC, and Lip), and T6 (immunodominant antigen B IsaB, Nuc, and degraded Geh). The protein pattern of HG002 was identical to that of RN1, confirming that the TcaR regulator plays a minor role in the regulation of exoproteins.

HG001 and HG003 (both strains are *rsbU* repaired) showed a completely different protein pattern compared to those of RN1 and HG002, illustrating that the restoration of *rsbU* and consequently full SigB activity have a major influence on the exoprotein pattern. The most prominent proteins were R1 (Lip), R2 (Lip and formyltetrahydrofolate synthetase Fhs), R3 (Atl and Lip), R4 (glycerophosphoryl diester phosphodiesterase GlpQ), and R5 (immunodominant antigen B IsaB). The different protein pattern cannot be explained only by protease activity, as in the *rsbU*-defective strains (RN1 and HG002), Geh (T4) and HlgB and HlgC (T5), new other proteins, were highly expressed.

In UAMS-1, protein A was identified in many bands (A2,

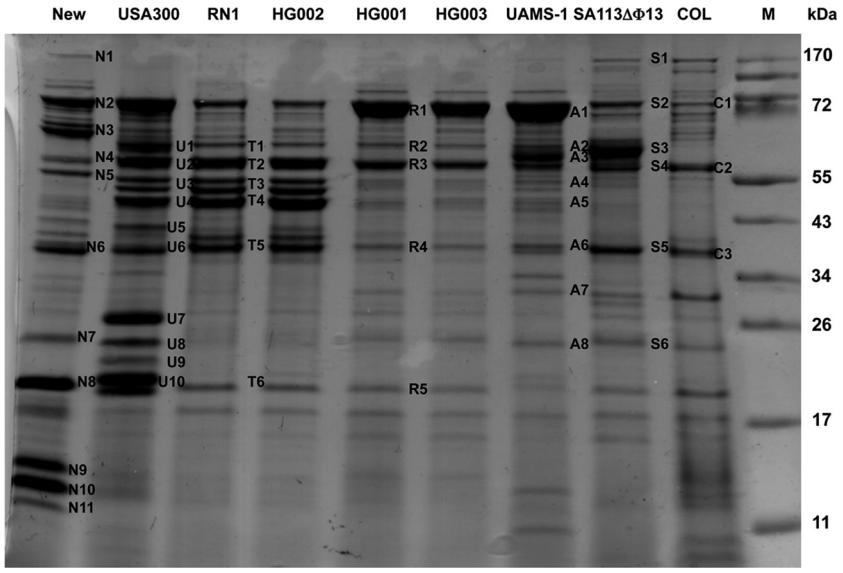

FIG. 5. Exoprotein pattern of the *S. aureus* strains from 7-h culture and MALDI-TOF analysis of various bands. For Newman (New), N1 (Atl), N2 (Lip, Atl), N3 (Coa, Lip, MapW), N4 (Atl, MapW, HP [NWMN_0687], Coa), N5 (Sbi), N6 (HlgA), N7 (Nuc), N8 (Efb), N9 (HP: Fib [NWMN_1066]), N10 (Efb), N11 (Efb). For USA300, U1 (Lip, Spa), U2 (Atl), U3 (Lip, Tuf [first band], Lip, Atl [second band]), U4 (Geh), U5 (LukF-PV), U6 (LukS-PV, Lip), U7 (HP [SAP003]), U8 (Nuc), U9 (Stp), U10 (Nuc, Stp, HP [SAUSA300_pUSA010004]). For RN1 and HG002, T1 (Lip), T2 (Atl, HP [SAOUHSC_00728]), T3 (Lip [first band], Lip [second band]), T4 (Geh), T5 (HlgB, HlgC, Lip), T6 (IsaB, Geh, Nuc). For HG001 and HG003, R1 (Lip), R2 (Lip, Fhs), R3 (Atl, Lip), R4 (GlpQ), R5 (IsaB). For UAMS-1, A1 (Lip, Geh), A2 (Lip, Spa), A3 (Lip, Spa), A4 (Lip, Spa [first band]), Lip, Spa [second band]), A5 (Lip, Geh), A6 (Lip, HlgA, HlgC, Spa), A7 (Lip, SsaA, CodY), A8 (Lip, Nuc, Stp). For SA113ΔΦ13, S1 (Atl), S2 (Geh), S3 (Spa), S4 (HP [SAOUHSC_00728]), S5 (GlpQ), S6 (HP, surface protein [SAOUHSC_00094]). For COL, C1 (Lip), C2 (HP [SACOL0778]), C3 (GlpQ). All *S. aureus* strains, with the exception of RN1 and HG002 as well as HG001 and HG003, showed different exoprotein expression patterns.

A3, A4, and A6), but the most dominant protein was Lip, which was present in many protein bands (A1 to A8), followed by Geh (A1, A4, and A5). A6 contains in addition to Lip gamma-hemolysin A and C, and band A7 is composed of a secretory antigen SsaA homolog as well as CodY, a transcriptional repressor. A8 was composed of Nuc and a truncated form of staphopain. We observed that typical cytoplasmic proteins were found in small amounts in the culture supernatants; this is a well-known phenomenon for most bacteria, which is explained by onset of autolysis during the exponential phase.

In SA113ΔΦ13, the most prominent protein bands were S1 (Atl), S2 (glycerol ester hydrolase Geh), S3 (Spa), S4 (HP, similar to sulfatase family protein), S5 (glycerophosphoryl diester phosphodiesterase GlpQ), and S6 (HP, similar to surface protein).

In COL, the most prominent proteins were C1 (Lip), C2 (HP, similar to sulfatase family protein), and C3 (glycerophosphoryl diester phosphodiesterase, GlpQ).

A processed form of Atl could be identified in large amounts in USA300 (U2), RN1, HG002 (T2), and HG001 and HG003 (R3). This protein overlaps with a protein that is similar to sulfatase family protein RN1 (T2), SA113ΔΦ13 (S2), and COL (C2).

We also show the results of the SDS-PAGE of extracellular proteins present at an early time point to illustrate the exceptional status of strain Newman (Fig. 6). Newman is the only strain that produced proteins in large amounts in the early exponential growth phase. There are three highly expressed proteins that were identified as coagulase (N12 and N13), the IgG-binding protein Sbi (N14), and Efb (extracellular fibrinogen-binding protein) (N15). Newman overproduced these proteins at all three tested time points, 2.5, 5 (not shown), and 7 h (not shown). This unique protein pattern is due to the point mutation in *saeS* (1) and was not shown by any of the other strains.

Biofilm formation. Our results are only partially consistent with the previous observation that *agr* mutants of *S. aureus*, as well as of *S. epidermidis*, exhibit a high propensity to form biofilms (7, 35, 43). Thus, in our biofilm assay, with polystyrene plates, the only strains that formed a robust biofilm carried the *agr* mutation, including SA113ΔΦ13 (*rsbU tcaR agr*), the RN6911 Δ*agr* mutant, and the spontaneous *agr* mutant of RN1 (data not shown) (Fig. **7**). However, COL, which has only low RNAIII transcript level and very likely has an *agr* defect, generated rather weak biofilm. The *agr*$^+$ strains, Newman and USA300, also generated weak biofilms, as expected, whereas the restoration of *rsbU* (HG001 and HG003) led to significant increases in biofilm formation (Fig. 7), even though these strains are both *agr*$^+$. RN1 and HG002 (*agr*$^+$) formed somewhat heavier biofilms than Newman and USA300, and there was no difference between these two strains, indicating that *tcaR* has no effect on biofilm formation. Genotypes other than *agr* are, of course, involved in biofilm formation, and the results presented here simply underline this fact, providing a further demonstration of the profound phenotypic variability among the strains under study.

Quantitative analysis of adhesion and biofilm formation on four polystyrene microtiter plates was determined with an enzyme-linked immunosorbent assay (ELISA) reader (450 nm). The mean values (standard deviations) were 0.075 (0.014) for

TABLE 3. List of identified exoproteins from 7-h (2.5-h) culture

Strain and protein band	N315 ORF[a]	Gene identifier	Gene	Protein function	SP[b]	MW (in thousands) of mature protein[c]	PSORTb localization
Newman							
N1	SA0905	NWMN_0922	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7	Extracellular
N2	SA0905	NWMN_0922	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7	Extracellular
	SA2463	NWMN_2569	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9	Extracellular
N3	SA0222	NWMN_0166	*coa*	Coagulase	+	71.8#	Extracellular
	SA2463	NWMN_2569	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA1751	NWMN_1872	*mapW*	Truncated *mapW* protein	+	50.3	Extracellular
N4	SA0905	NWMN_0922	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7#	Extracellular
	SA1751	NWMN_1872	*mapW*	Truncated *mapW* protein	+	50.32	Extracellular
	SA0674	NWMN_0687		Hypothetical protein, sulfatase family protein	+	71	Membrane
	SA0222	NWMN_0166	*coa*	Coagulase	+	71.8#	Extracellular
N5	SA2206	NWMN_2317	*sbi*	IgG-binding protein Sbi	+	47	Unknown
N6	SA2207	NWMN_2318	*hlgA*	Gamma-hemolysin chain II	+	31.9	Extracellular
N7	SA0746	NWMN_0760	*nuc*	Staphylococcal nuclease (thermonuclease)	+	18.8	Extracellular
N8	SA1003	NWMN_1069	*efb*	Extracellular fibrinogen-binding protein	+	15.9	Extracellular
N9	SA1000	NWMN_1066		Hypothetical protein, similar to fibrinogen-binding protein	+	9.6	Extracellular
N10	SA1003	NWMN_1069	*efb*	Extracellular fibrinogen-binding protein	+	15.9#	Extracellular
N11	SA1003	NWMN_1069	*efb*	Extracellular fibrinogen-binding protein	+	15.9#	Extracellular
N12	SA0222	NWMN_0166	*coa*	Coagulase	+	71.8	Extracellular
N13[d]	SA0222	NWMN_0166	*coa*	Coagulase	+	71.8	Extracellular
N14[d]	SA2206	NWMN_2317	*sbi*	IgG-binding protein Sbi	+	47	Unknown
N15[d]	SA1003	NWMN_1069	*efb*	Extracellular fibrinogen-binding protein	+	15.9	Extracellular
USA300							
U1	SA2463	SAUSA300_2603	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0107	SAUSA300_0113	*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
U2	SA0905	SAUSA300_0955	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7#	Extracellular
U3[1.band]	SA2463	SAUSA300_2603	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0506	SAUSA300_0533	*tuf*	Elongation factor Tu	−	43	Cytosolic
U3[2.band]	SA2463	SAUSA300_2603	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0905	SAUSA300_0955	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7#	Extracellular
U4	SA0309	SAUSA300_0320	*geh*	Glycerol ester hydrolase	+	72.4#	Extracellular
U5	SA1637	SAUSA300_1381	*lukF-PV*	Panton-Valentine leukocidin, LukF-PV	+	34.5	Extracellular
U6	SA2208	SAUSA300_1382	*lukS-PV*	Panton-Valentine leukocidin, LukS-PV	+	32.3	Extracellular
	SA2463	SAUSA300_2603	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
U7	SAP003			Hypothetical protein	+	23.1	Extracellular
U8	SA0746	SAUSA300_0776	*nuc*	Staphylococcal nuclease (thermonuclease)	+	18.8	Extracellular
U9	SA1725	SAUSA300_1890	*stp*	Staphopain, cysteine proteinase	+	41.5#	Extracellular
U10		SAUSA300_pUSA010004		Hypothetical protein	+	17.2	Extracellular
	SA0746	SAUSA300_0776	*nuc*	Staphylococcal nuclease (thermonuclease)	+	18.8	Extracellular
	SA1725	SAUSA300_1890	*stp*	Staphopain, cysteine proteinase	+	41.5#	Extracellular
RN1 (NCTC8325)							
T1	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
T2	SA0905	SAOUHSC_00994	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7#	Extracellular
	SA0674	SAOUHSC_00728		Hypothetical protein, sulfatase family protein	+	71#	Membrane
T3[1.band]	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
T3[2.band]	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
T4	SA0309	SAOUHSC_00300	*geh*	Glycerol ester hydrolase	+	72.4#	Extracellular
T5	SA2209	SAOUHSC_02710	*hlgB*	Gamma-hemolysin component B	+	33.9	Extracellular
	SA2208	SAOUHSC_02709	*hlgC*	Gamma-hemolysin component C	+	32.5	Extracellular
	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular

T6	SA2431	SAOUHSC_02972	*isaB*	Immunodominant antigen B	+	15.8	Extracellular
	SA0309	SAOUHSC_00300	*geh*	Glycerol ester hydrolase	+	72.4#	Extracellular
	SA0746	SAOUHSC_00818	*nuc*	Staphylococcal nuclease (thermonuclease)	+	18.8	Extracellular
HG001							
R1	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9	Extracellular
R2	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA1553	SAOUHSC_01845	*fhs*	Formyltetrahydrofolate synthetase		60	Cytosolic
R3	SA0905	SAOUHSC_00994	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7#	Extracellular
	SA2463	SAOUHSC_03006	*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
R4	SA0820	SAOUHSC_00897	*glp*Q	Glycerophosphoryl diester phosphodiesterase	+	32.2	Extracellular
R5	SA2431	SAOUHSC_02972	*isaB*	Immunodominant antigen B	+	15.8	Extracellular
UAMS-1							
A1	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9	Extracellular
	SA0309		*geh*	Glycerol ester hydrolase	+	72.4	Extracellular
A2	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0107		*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
A3	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0107		*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
$A4^{1.band}$	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0107		*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
	SA0309		*geh*	Glycerol ester hydrolase	+	72.4#	Extracellular
$A4^{2.band}$	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0107		*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
A5	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0309		*geh*	Glycerol ester hydrolase	+	72.4#	Extracellular
A6	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA2207		*hlgA*	Gamma-hemolysin chain II precursor	+	31.9	Extracellular
	SA2208		*hlg*C	Gamma-hemolysin component C	+	32.5	Extracellular
	SA0107		*spa*	Immunoglobulin G-binding protein A	+	45.3#	Cell wall
A7	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA2093		*ssaA*	Secretory antigen precursor SsaA homolog	+	26.7	Extracellular
	SA1098		*codY*	Transcriptional repressor CodY		28.8	Cytosolic
A8	SA2463		*lip*	Triacylglycerol lipase precursor, glycerol ester hydrolase 1	+	72.9#	Extracellular
	SA0746		*nuc*	Staphylococcal nuclease (thermonuclease)	+	18.8	Extracellular
	SA1725		*stp*	Staphopain, cysteine proteinase	+	41.5#	Extracellular
SA113ΔΦ13							
S1	SA0905	SAOUHSC_00994	*atl*	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	+	133.7	Extracellular
S2	SA0309	SAOUHSC_00300	*geh*	Glycerol ester hydrolase	+	72.4	Extracellular
S3	SA0107	SAOUHSC_00069	*spa*	Immunoglobulin G-binding protein A	+	45.3	Cell wall
S4	SA0674	SAOUHSC_00728		Hypothetical protein, sulfatase family protein	+	71#	Membrane
S5	SA0820	SAOUHSC_00897	*glpQ*	Glycerophosphoryl diester phosphodiesterase	+	32.2	Extracellular
S6	SA0129	SAOUHSC_00094		Hypothetical protein, surface protein	+	23.6	Cell wall
COL							
C1	SA2463	SACOL2694	*geh*	Glycerol ester hydrolase	+	72.4	Extracellular
C2	SA0674	SACOL0778		Hypothetical protein, sulfatase family protein	+	71#	Membrane
C3	SA0820	SACOL0962	*glpQ*	Glycerophosphoryl diester phosphodiesterase	+	32.2	Extracellular

[a] ORF, open reading frame.
[b] SP, signal peptide.
[c] MW was calculated (http://www.expasy.org/tools/pi_tool.html) without signal sequence if there was one predicted (http://www.cbs.dtu.dk/services/SignalP). #, probably fragment of the mature protein.
[d] 2.5-h culture.

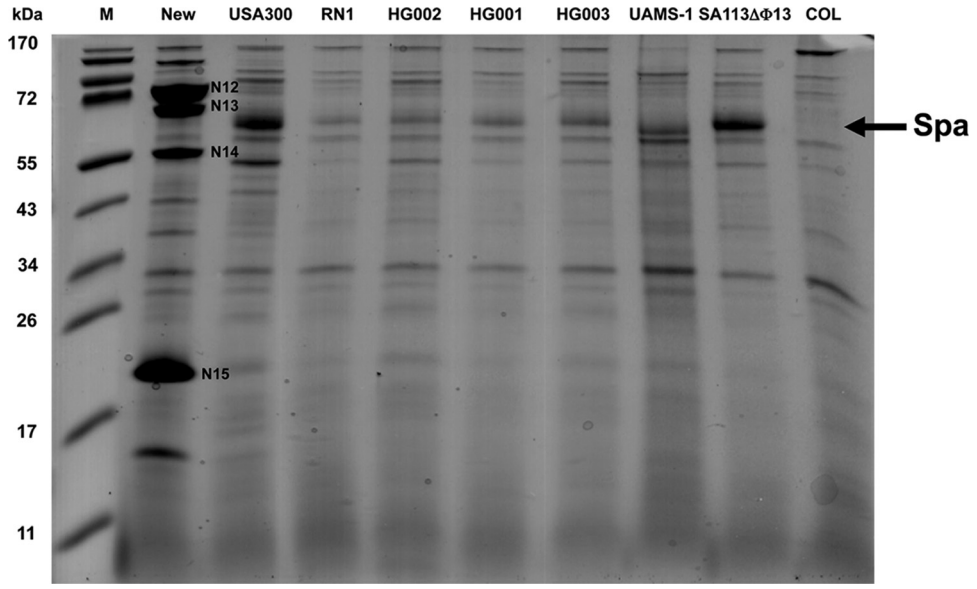

FIG. 6. Exoprotein pattern of a 2.5-h culture and MALDI-TOF analysis of prominent bands of Newman. Because of the unique protein pattern, the focus here is on the Newman strain. For Newman, N12 (Coa), N13 (Coa), N14 (Sbi), N15 (Efb).

Newman, 0.108 (0.086) for USA300, 0.193 (0.060) for RN1, 0.203 (0.065) for HG002, 0.335 (0.052) for HG001, 0.335 (0.043) for HG003, 0.097 (0.037) for UAMS-1, 0.473 (0.018) for SA113ΔΦ13, and 0.118 (0.012) for COL. The results were essentially the same as those shown in Fig. 7.

Comparative virulence in a mouse sepsis model. We used a mouse sepsis model to compare the virulence of the nine *S. aureus* strains RN1, HG002, HG001, HG003, Newman, USA300, UAMS-1, and COL. Mice were challenged with 5×10^8 and 1×10^8 CFU. The results revealed significant differences in mouse lethality among these nine strains (Fig. 8). Survival was significantly lower for animals infected with 5×10^8 CFU of strains Newman, USA300, HG001, and HG003 than with strains UAMS-1, RN1, HG002, and COL. HG003 and HG001 were less virulent after infection with 5×10^8 CFU than strain Newman ($P = 0.046$) but were not significantly less virulent than strain USA300 ($P = 0.523$). Although strain Newman showed a slightly higher mortality after infection with 5×10^8 CFU than USA300 (100% mortality at 24 h versus 83% mortality at 24 h), the difference between both strains was not significant ($P = 0.093$). HG001 and HG003 showed significantly higher virulence than UAMS-1 ($P = 0.002$), RN1 ($P = 0.005$), HG002 ($P = 0.005$), or COL ($P = 0.001$).

Likewise, infection with a lower dose (1×10^8 CFU) resulted in increased survival of animals after infection with strain Newman (0% after 72 h), HG003 (16% after 120 h), USA300 (16% after 120 h), HG001 (40% after 120 h), and UAMS-1 (33% after 120 h) compared to strains HG002 (50% after 120 h), RN1 (33% after 120 h), and COL (100% after 120 h).

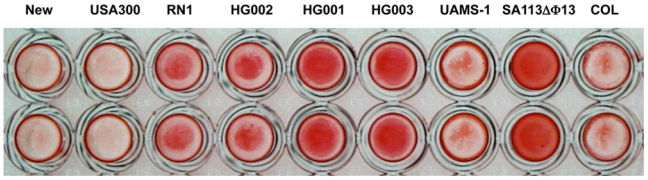

FIG. 7. Biofilm formation in microtiter plates. Strains tested were *S. aureus* RN1 and derivatives HG002, HG001, HG003, and SA113ΔΦ13 and the clinical isolates Newman, USA300, UAMS-1, and COL.

Notably, animals infected with HG003 and HG001 died more rapidly than animals infected with HG002 or RN1 at either dose, suggesting a positive role of a functional *sigB* regulon on virulence of HG003 and HG001 in the sepsis model. However, this difference was not significant at the lower dose ($P = 0.092$). HG003 showed no significantly reduced virulence at 1×10^8 compared to Newman ($P = 0.123$), USA300 ($P = 0.713$), and RN1 ($P = 0.137$). In contrast, HG003 was more virulent than UAMS-1 ($P = 0.043$) and COL ($P = 0.005$). Infection with strain COL resulted in a very low mortality, which is in accordance with previous studies reporting the low virulence potential of this early MRSA isolate. We also tested SA113ΔΦ13 (not shown), which showed a low virulence similar to that of COL, very likely due to its treatment with mutagens to obtain a restriction-deficient variant.

In summary, the comparative virulence studies revealed that, depending on the infection dose, there are significant differences in the virulence potentials. Combining all data, the following order of virulence in the mouse sepsis model became evident: Newman was the most virulent, followed by USA300, HG003, HG001, UAMS-1, HG002, and RN1; the least virulent strain was COL.

DISCUSSION

This study was undertaken to address the important question of what influence global regulators in *S. aureus* have on various phenotypes and pathobiology. We would first point out that all of the generic strains presently under study, of which several representative examples have been examined in this

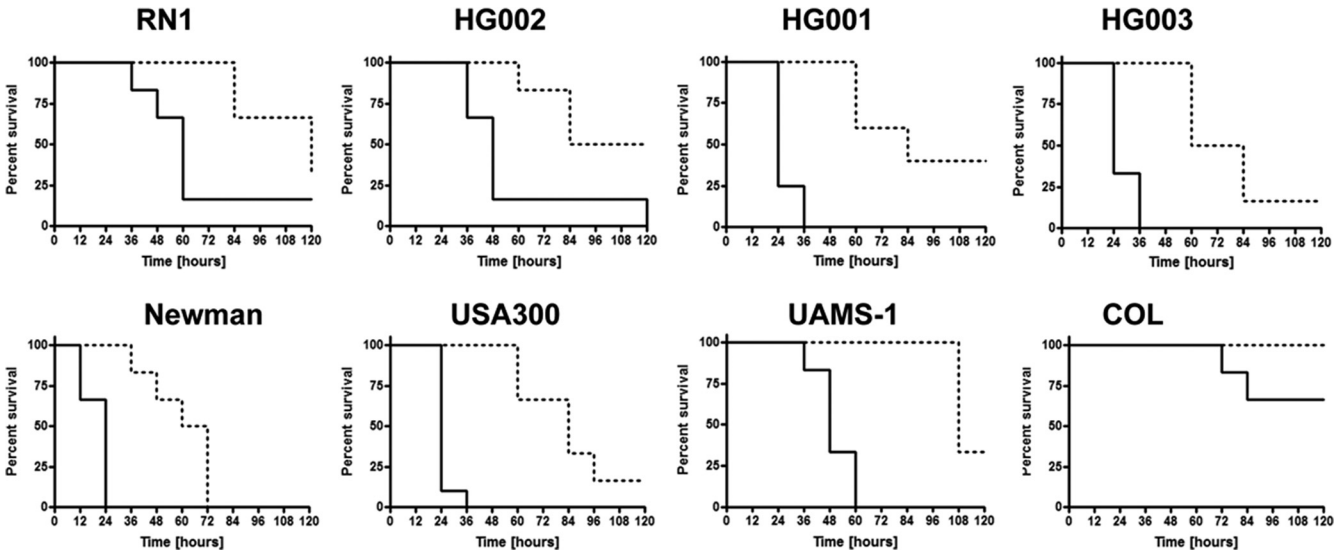

FIG. 8. Virulence of *S. aureus* strains in a mouse sepsis model. Kaplan-Meier curves indicate survival of mice after infection with 5×10^8 (solid line) and 1×10^8 CFU (dashed line) via the tail vein, using 6 mice per group.

report, are primary human infection isolates. This means that all are virulent for humans and are therefore appropriate for studies that relate to human disease. Although these isolates vary widely in animal virulence, there is no way to determine whether this variation correlates with variation in human virulence; therefore, relative animal virulence is probably not a valid criterion for choosing one strain over another. Since there is now a very large body of data, derived from genome sequencing, regulatory analyses, determination of metabolic and catabolic capacities, content and expression of genes involved in pathogenesis, etc., such a choice could be made with some confidence. However, a major complication, at this theoretical level, would be the content and nature of mobile genetic elements, which not only vary widely and contribute substantively to an organism's biotype or pathotype but can be readily exchanged among strains and thus tend to confound the concept of a "generic" strain. Nevertheless, our studies do tend to underline the potential utility of repaired NCTC8325 for this role.

In the characterization of strains presented here, a number of interesting features have come to light. First, these studies have revealed great interstrain variations in phenotype. Second, most of these strains possess one or more regulatory features by which they differ from the others. Thus, all differ in their regulatory genotypes. Newman carries a missense mutation in *saeS*, resulting in a complex pattern of virulence gene regulation that is quite different from that of strains with the wild-type (wt) *saeS* gene (1); UAMS-1 contains a deletion in *sarT* and *sarU*, genes involved in *agr* regulation; N315 has a deletion in *arlR*, a global regulator of autolysins and of *agr* expression (26), and is also *agr* defective, owing to a mutation in *agrC*; COL is significantly attenuated in virulence owing to an *agr* defect and possibly to other unknown regulatory mutations. USA300 has a unique regulatory phenotype that sets it apart from the more typical strains, namely, simultaneous overproduction of both Hla and Spa. Genome analyses suggest that there are "default" regulatory genotypes and that most of the strains under study differ from these defaults, suggesting that the deviations are mutational. This would probably rule out all but one of them as the generic choice, namely, NCTC8325, which is enormously useful because of the incomparable wealth of knowledge that has accumulated in the almost 50 years since its isolation and because of the vast number of derivative strains that are highly useful for any studies involving manipulation of staphylococcal genes. At the same time, NCTC8325 has mutational defects in two regulatory genes, *rsbU* and *tcrA*. In the interest of retaining the enormously useful body of knowledge plus the vast collection of derivative strains, three different labs have repaired the *rsbU* defect, clearly the more important of the two. In the first two cases (20, 38), derivatives that had previously been cured of the strain's three resident prophages were used. For unknown reasons these cured strains are somewhat unstable; additionally, the 3 prophages should be considered significant components of the genome, which should ideally be left intact in any "generic" strain. We note, for example, that the prophages in Newman contribute to pathogenicity independently of any defined virulence-converting genes (3). Accordingly, we have also repaired the *rsbU* defect and have additionally repaired the *tcaR* mutation. The former, as was well-known, had a profound effect on phenotype, so that the repair has brought it largely into line with the generic phenotype, whereas the latter had at most a rather minor effect, mainly on *spa* regulation. A second candidate strain is Newman, currently in wide use because it is quite virulent for mice, has a functional SigB system, and has been used as the host strain for a large and important transposon library (4). Again, however, Newman has a rather serious regulatory mutation in *saeS* (36), which has a very considerable impact on its phenotype, shown most clearly in Fig. 6, though its effect, if any, on virulence is unknown. The *saeS* defect was complemented by a plasmid-encoded wild-type gene, which resulted in a drastic decrease of exoproteins (1). Very recently, the *saeS* defect was also repaired in the chromosome using the wt gene from RN1. The repaired strain

(NewHG) was severely affected in production of extracellular proteins, such as coagulase, Sbi, gamma-hemolysin, and fibrinogen-binding protein (27). NewHG and HG003 could be satisfactory standard strains to verify results obtained with other strains. Because of its functional global regulators, HG003 might be particularly helpful in studying questions that address regulation. However, it should be emphasized that there is no single optimal strain of *S. aureus* for studying molecular genetics and pathobiology. Most clinical isolates differ from each other more or less. It is assumed that in the course of colonization or infection, a permanent strain adaptation and selection occur, resulting in an enhanced potential to resist the immune response, to colonize, to spread after initial adherence, and to multiply in different organs. To unravel the ongoing evolutionary steps during infection and the characterization of such isolates or of epidemic strains, like USA300, is of extreme clinical importance. This study provides a helpful basis to interpret the phenotype of new clinical isolates.

Another consideration that we have not addressed is host specificity. Although it is clearly desirable to focus on human-adapted strains, especially for studies of pathogenicity, and to use the mouse, where possible, as an experimental animal, this combination is problematic because human-adapted strains would not be expected to perform in a parallel manner in mouse models. For example, mouse neutrophils are not susceptible to PVL toxin (19), a disparity that probably underlies the recent controversy over the role of PVL in CA-MRSA pathogenesis (41, 42). Additionally, it is well-known that mice have a naturally low susceptibility to bacterial toxins (33) and superantigens, like toxic shock syndrome toxin 1 (TSST-1); the susceptibility can be increased, however, by pretreatment with endotoxin (37). For these reasons, different strains are appropriately used for certain infection models, and it could be argued that strains naturally adapted to these animals should be used when possible. Alternatively, a generic strain could be animal adapted, but this would not be likely to mimic the natural adaptation.

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